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ORIGINAL ARTICLE

# GABA<sub>B</sub> receptor allosteric modulators exhibit pathway-dependent and species-selective activity

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## Keywords

Allosteric modulator, biased signaling, functional selectivity, GABA<sub>B</sub> receptor, ortholog selectivity

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## Abstract

Positive modulation of the GABA<sub>B</sub> receptor (GABA<sub>B</sub>R) represents a potentially useful therapeutic approach for the treatment of nicotine addiction. The positive allosteric modulators (PAMs) of GABA<sub>B</sub>R GS39783 and BHF177 enhance GABA-stimulated [<sup>35</sup>S]GTPγS-binding, and have shown efficacy in a rodent nicotine self-administration procedure reflecting aspects of nicotine dependence. Interestingly, the structural related analog, NVP998, had no effect on nicotine self-administration in rats despite demonstrating similar pharmacokinetic properties. Extensive in vitro characterization of GS39783, BHF177, and NVP998 activity on GABA<sub>B</sub>R-regulated signaling events, including modulation of cAMP, intracellular calcium levels, and ERK activation, revealed that these structurally related molecules display distinct pathway-specific signaling activities that correlate with the dissimilarities observed in rodent models and may be predictive of in vivo efficacy. Furthermore, these GABA<sub>B</sub>R allosteric modulators exhibit species-dependent activity. Collectively, these data will be useful in guiding the development of GABA<sub>B</sub>R allosteric modulators that display optimal in vivo efficacy in a preclinical model of nicotine dependence, and will identify those that have the potential to lead to novel antismoking therapies.

## Abbreviations

cAMP, cyclic adenosine monophosphate; CHO cells, Chinese hamster ovary cells; CRC, concentration–response curve; ERK, Extracellular signal-regulated kinases; GABA, γ-aminobutyric acid; GPCR, G protein-coupled receptor; HEK293 cells, human embryonic kidney 293 cells; PAM, Positive allosteric modulator.

## Introduction

The GABA<sub>B</sub>R is an obligate heterodimer, where heterodimerization between the GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2 subunits form the functional GABA<sub>B</sub>R. The GABA<sub>B</sub>R1 subunit contains the  $\gamma$ -aminobutyric acid (GABA) orthosteric agonist binding site, whereas the GABA<sub>B</sub>R2 subunit couples to heterotrimeric G<sub>i/o</sub> proteins. Agonism of the GABA<sub>B</sub>R leads to a decrease in cAMP levels via inhibition of adenylyl cyclase, increased intracellular calcium (New *et al.* 2006), and phosphorylation of the extracellular-signal regulated protein kinase 1/2 (ERK<sub>1/2</sub>) (Tu *et al.* 2007) (Vanhoose *et al.* 2002). Thus, GABA<sub>B</sub>R couples to several distinct intracellular signal transduction pathways. Dysfunction of GABA<sub>B</sub>R signaling is associated with various disorders including epilepsy, anxiety, depression, sleep disorders, cognitive impairment, and drug addiction (Pinard *et al.* 2010). Hence, GABA<sub>B</sub>R agonists, such as baclofen, are of potential utility in the management of such diseases. However, side effects, principally sedation, tolerance, and motor impairment, limit their therapeutic use.

Emerging concepts in receptor pharmacology, such as functional selectivity and allosteric modulation, have attracted considerable interest recently from a therapeutic perspective. Functional selectivity or ligand-bias refers to the ability of a particular ligand to activate one signaling pathway to the exclusion of others suggesting that one can fine-tune the signaling output of a target receptor. Allosteric modulation arises from molecules that bind to a site on the receptor that is topographically distinct from the orthosteric binding site of the receptor's natural ligand (Christopoulos 2002). Allosteric ligands modulate not only orthosteric ligand efficacy but can also display intrinsic activity. The discovery of positive allosteric modulators (PAMs) of the GABA<sub>B</sub>R, was first reported by Urwyler *et al.* (2001). CGP7930 was found to specifically act on the GABA<sub>B</sub>R2 subunit, yet it exhibited intrinsic agonist activity suggesting allosteric communication between subunits (Binet *et al.* 2004). In preclinical studies, CGP7930 in combination with baclofen has shown improved therapeutic-like benefits over baclofen alone with respect to sedation (Carai *et al.* 2004), depression-like (Mombereau *et al.* 2004), and drug-seeking behaviors in rodents (Lhuillier *et al.* 2007; Mombereau *et al.* 2007). High throughput screening of small molecule libraries led to the identification of the positive allosteric modulator N'-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidin-4,6-diamine (GS39783) (Urwyler *et al.* 2003, 2005; Guery *et al.* 2007). This chemical scaffold was led to the development of analogs BHF177 and NVP998 which have been demonstrated to

enhance GABA-dependent GABA<sub>B</sub>R activation (Guery *et al.* 2007). Subsequently, it was shown that BHF177 was effective at decreasing nicotine self-administration, supporting the potential utility of GABA<sub>B</sub>R PAMs as novel pharmacotherapies for nicotine addiction. It should be noted here that nicotine is the main psychoactive ingredient in tobacco that is believed to perpetuate the harmful tobacco smoking. Interestingly, it was found that GS39783 could only achieve comparable *in vivo* efficacy as BHF177 when co-administered with a subeffective dose of the GABA<sub>B</sub>R agonist CGP44532 (Paterson *et al.* 2008).

Here, we show that the structurally related GS39783/BHF177 analog NVP998 failed to decrease nicotine self-administration in the rat despite having similar *in vivo* pharmacokinetic properties. To test whether the dissimilarities observed *in vivo* could be explained by differences in the *in vitro* pharmacological activity profile of these allosteric modulators, we developed a multiple cell-based functional assay platform to investigate the effects of GS39783, BHF177, and NVP998 on GABA<sub>B</sub>R-mediated cellular responses including cAMP production, intracellular Ca<sup>2+</sup> mobilization, and ERK<sub>1/2</sub> phosphorylation. The modulation of GABA<sub>B</sub>R-mediated cellular responses was investigated using both rat and human GABA<sub>B</sub>R-expressing cellular systems. Collectively, our results reveal the existence of functional selectivity and species-selective modulation of GABA<sub>B</sub>R signaling by these structurally related GABA<sub>B</sub>R allosteric modulators.

## Materials and methods

### Intravenous nicotine self-administration

#### Animals

Male Wistar rats (Charles River, Raleigh, NC), weighing 300–350 g at the beginning of the experiments, were housed two per cage in an environmentally controlled vivarium on a reversed 12 h/12 h light/dark cycle throughout the experiment. All behavioral testing occurred during the dark phase of the light/dark cycle. Rats had *ad libitum* access to food and water initially and were subsequently put on a food-restricted diet of 20 g rat chow per day during behavioral training and testing. All procedures were conducted in accordance with the guidelines from the National Institutes of Health and the Association for the Assessment and Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committees of our institutions.

## Apparatus

Standard operant conditioning chambers (24 × 30 × 28 cm; Med Associates, St. Albans, VT) were used in all experiments as described previously (Liechti *et al.* 2007a).

## Food self-administration under a fixed-ratio schedule of reinforcement

Naive rats were gradually trained to lever press for food (45 mg Noyes food pellets) on a fixed-ratio five timeout 20 sec (FR5 TO20 s) schedule during 1 h sessions for 3 weeks (5 days per week). After successful acquisition of stable food self-administration, NVP998 (0, 20, 40 and 80 mg/kg, *p.o.*; 1 h pretreatment) was administered using a within-subjects Latin-square design. At least 7 days elapsed between drug administrations, and rats were required to exhibit stable baseline performance prior to drug administration.

## Nicotine self-administration under a fixed-ratio schedule of reinforcement

Naive rats were gradually trained to lever press for food on an FR5 TO20 s schedule over a period of 5–7 days. After food training, all the rats were surgically prepared with an intravenous catheter inserted into the right jugular vein under isoflurane (1–3% in oxygen) anesthesia as described previously (Liechti and Markou 2007b). For the rats used for the assessment of intracerebroventricular (*i.c.v.*) administration of NVP998 on nicotine self-administration, guide cannulae (26 gage; Plastics One, Roanoke, VA) were implanted bilaterally 1 mm above the cerebroventricular (from bregma: anterior/posterior, 0.9 mm; medial/lateral, ±1.8 mm; dorsal/ventral, −3.4 mm from dura; angle: 10 degree) using a stereotaxic frame (David Kopf Instruments, Tujunga, CA). After surgery, the animals were allowed to recover for at least 5 days before nicotine self-administration training was initiated. During nicotine self-administration training, animals self-administered nicotine (0.03 mg/kg/infusion) on an FR5 TO20 s schedule during 1 h sessions for 4–5 weeks (5 days per week). After successful acquisition of stable nicotine self-administration, NVP998 (0, 20, 40, and 80 mg/kg, *p.o.*; 1 h pretreatment) was administered using a within-subjects Latin-square design. At least 7 days elapsed between drug administrations, and rats were required to exhibit stable baseline performance prior to drug administration. For the rats that were prepared with intracranial guide cannulae, NVP998 (0, 4 and 8 µg/side, 0.5 h pretreatment) was administered using a between-subjects design.

## Nicotine self-administration under a progressive-ratio schedule of reinforcement

The food training, intravenous catheterization surgery, and nicotine self-administration training under the fixed-ratio schedule were the same as that described in section 2.7.4. After successful acquisition of stable nicotine self-administration under fixed-ratio, rats were switched to the progressive-ratio schedule of reinforcement as described previously (Paterson *et al.* 2008). In this schedule, the level press numbers for a single nicotine infusion were progressively increased. The break-point was defined as the highest ratio completed before the first 1 h period during which no injections were earned. The number of responses required to earn a nicotine infusion on the progressive ratio was determined by the exponential progression [ $5e^{(0.25 \times (\text{infusion number} + 3))} - 5$ ], with the first two values replaced by 5 and 10 (modified from (Richardson and Roberts 1996)), so that the response requirements for successive reinforcers were 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, etc. The effects of NVP998 (0, 20, 40 and 80 mg/kg, *p.o.*; 1 h pretreatment) were assessed in animals that showed stable nicotine self-administration behavior using a within-subjects Latin-square design.

## Pharmacokinetic procedures

To assure that differences in the self-administration tests were not due to pharmacokinetics or CNS exposure, rats were administered 20 mg/kg GS39783, BHF177, or NVP998 by oral gavage and plasma levels were quantitated at 0.5, 1, and 1.5 h. These times represented the beginning, middle, and end of the self-administration experiments where compound was administered 30 min prior to initiating the testing session. Brains were collected at the 1.5 h time point. Plasma was generated by standard centrifugation techniques and immediately frozen. Plasma and brain were mixed with acetonitrile (1:5 *v:v* or 1:5 *w:v*, respectively). Plasma samples were vortexed and allowed to sit on ice for 15 min, and brain samples were disrupted with a probe tip sonicator. Samples are centrifuged through a Millipore Multiscreen Solvinter 0.45 micron low binding PTFE hydrophilic filter plate and drug levels were determined using an ABSciex 5500 mass spectrometer (Framingham, MA, U.S.A.) with multiple reaction monitoring and mass transitions of 338.2→202.1 for GS39783, 348.2→254.2 for BHF177, and 359.2→265.2 for NVP998. Brain concentrations were quantitated as drug per mg tissue and converted to a molar concentration by assuming a conversion of 1 g tissue equals 1 mL.

## Compounds

GABA, baclofen, CGP54626, and GS39783 were purchased from Tocris Biosciences (Avonmouth, Bristol, United Kingdom). BHF177 and NVP998 were synthesized and provided by Dr. M.G. Finn, Georgia Tech, GA. (–) Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in saline (0.9%) and pH-adjusted to 7.4 ( $\pm 0.5$ ) with 1 mol/L sodium hydroxide solution. Nicotine doses are reported as freebase concentrations.

## Cell culture

A stable CHO (Chinese hamster ovary) cell line expressing a functional GABA<sub>B</sub>R comprised of human GABA<sub>B</sub>R1b and rat GABA<sub>B</sub>R2 (CHO<sub>RGBR</sub>; a kind gift from Dr. K. Kaupmann, Novartis, Basel, Switzerland), a stable CHO cell line expressing a functional GABA<sub>B</sub>R comprised of human GABA<sub>B</sub>R1b and human GABA<sub>B</sub>R2 (CHO<sub>HGBR</sub>) and a stable HEK293 (human embryonic kidney) cell line expressing a functional GABA<sub>B</sub>R comprised of human GABA<sub>B</sub>R1b and human GABA<sub>B</sub>R2 (HEK<sub>HGBR</sub>) were used for all cell-based experiments described herein. CHO<sub>RGBR</sub>, CHO<sub>HGBR</sub>, and HEK<sub>HGBR</sub> cells were cultured in F12 medium and DMEM, respectively, supplemented with 10% fetal bovine serum, 1 mg/mL geneticin, and 250  $\mu$ g/mL zeocin.

## Measurement of cAMP levels

CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells were seeded into white-walled 384-well plates at a density of 2000 and 2500 cells per well, respectively, in 20  $\mu$ L of growth media and cultured overnight at 37°C, 5% CO<sub>2</sub>. The following day medium was removed and replaced with 20  $\mu$ L of Hanks' balanced salt solution (HBSS), 20 mmol/L HEPES, pH 7.4. After 1 h incubation at room temperature (RT), 5  $\mu$ L of induction medium (HBSS, 20 mmol/L HEPES containing forskolin; 50  $\mu$ mol/L and 150  $\mu$ mol/L for CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub>, respectively) (MP biochemical, Irvine, CA 92618 USA), and the test compounds together with GABA were added to the cells at the indicated concentrations. After 10 min and 20 min incubations at RT for the CHO<sub>RGBR</sub> and the HEK<sub>HGBR</sub> cells, respectively, cAMP levels were measured using Cisbio' (Bedford, MA 01730 USA) cAMP dynamic HTRF assay kit as per the manufacturer's instructions. Briefly, induction medium was removed and the cells were lysed in 10  $\mu$ L of 1:1 PBS-lysis buffer containing cAMP labeled with the acceptor dye d2. Subsequently, 10  $\mu$ L of 1:1 PBS-lysis buffer containing cAMP-specific antibody labeled with cryptate was added to each well. After 1 h at room temperature, the amount of cAMP in lysate samples was quantified according to the manufacturer's instructions using the Envision plate reader (PerkinElmer, Shelton, CT 06484-4794

USA). Concentration–response curves (CRC) were recorded with four wells per concentration and experiment. The effects of the compounds were calculated relative to the stimulation obtained with a maximally active concentration of GABA. CRCs were determined by nonlinear regression analysis using Prism software (GraphPad Software Inc., San Diego, CA).

## Measurement of intracellular calcium concentration

CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells were seeded into black-walled 384-well plates at a density of 10,000 and 20,000 cells per well, respectively, in 20  $\mu$ L of growth media and cultured overnight at 37°C, 5% CO<sub>2</sub>. The following day the medium was removed and replaced with 20  $\mu$ L of loading medium consisting of HBSS, 20 mmol/L HEPES, 2.5 mmol/L probenecid, and fluorescent indicator Fluo-4 AM (Invitrogen, Carlsbad, CA 92008 USA). After 1 h incubation, cell and compound plates were placed into the fluorescence imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA 94089 USA). Compounds (prepared as 5 $\times$  solution in HBSS and 20 mmol/L HEPES, pH 7.4) were added at time = 10 sec and changes in fluorescence were monitored over a period of 250 sec following excitation at a wavelength of 488 nm and detection at 510–560 nm. Relative changes over baseline ( $\Delta F/F$ ) were determined. Concentration–response curves were recorded with four wells per concentration and experiment. The effects of compounds were calculated relative to the stimulation obtained with a maximally active concentration of GABA. CRCs were determined by nonlinear regression analysis using Prism software (GraphPad Software Inc., San Diego, CA).

## In cell ERK<sub>1/2</sub> phosphorylation Western blot

To determine the time point at which maximum ERK<sub>1/2</sub> phosphorylation occurs after GABA<sub>B</sub>R activation, CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells were seeded at 5000 cells/well in a 384-well plate and incubated overnight at 37°C, 5% CO<sub>2</sub>. The next day the medium was removed and replaced with 20  $\mu$ L of HBSS, 20 mmol/L HEPES. After 1 h incubation at 37°C, 5% CO<sub>2</sub>, 5  $\mu$ L of HBSS 20 mmol/L HEPES containing GABA was added to the plate and incubated at room temperature for the indicated time periods. After incubation, the medium was discarded and the cells were fixed in 4% paraformaldehyde in PBS for 20 min. Cells were then washed 3 $\times$  with PBS. After discarding the last wash, cells were permeabilized for 20 min with 0.2% Triton X-100 in PBS then washed 3 $\times$  in PBS. After discarding the last wash, Odyssey blocking buffer was added and the plate was rocked for 1 h at room temperature. Odyssey blocking buffer was then removed, and rabbit anti-phospho-ERK (1/150 in Odyssey blocking buffer) and mouse



anti-ERK (1/200 in Odyssey blocking buffer) were added to the plate and incubated overnight at 4°C. The following day the plates were washed three times with 0.1% Tween 20 in PBS. Infrared probe-labeled goat-anti-mouse and anti-rabbit (1/200 in 0.025% Tween 20 in PBS) were added. The plates were incubated for 1 h at room temperature, washed with 0.025% Tween 20, and scanned by the Odyssey infrared scanner. Data were acquired using the scanner software, and analyzed using Prism software (GraphPad Software Inc., San Diego, CA).

### Measurement of ERK<sub>1/2</sub> phosphorylation

CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells were seeded at 4000 cells/well in a 384-well plate and incubated overnight at 37°C, 5% CO<sub>2</sub>. The following day medium was removed and replaced with 20 µL of HBSS, 20 mmol/L HEPES, pH 7.4. After 1 h incubation, 5 µL of HBSS, 20 mmol/L HEPES containing GABA in the presence or absence of test compounds was added to the wells and incubated at room temperature for 5 min. After incubation, the medium was discarded and the cells were lysed in 16 M lysis buffer provided in the Cellul'erk HTRF cell-based assay (Cisbio). After 10 min of incubation, 4 µL of detection buffer containing the HTRF conjugates was added in each well. After 2 h at room temperature, the amount of phospho-ERK in the lysate samples was quantified according to the manufacturer's instruction using the Envision plate reader (PerkinElmer). Concentration–response curves were recorded with four wells per concentration and experiment. The effects of compounds were calculated relative to the stimulation obtained with a maximally active concentration of GABA. CRCs were determined by nonlinear regression analysis using Prism software (GraphPad Software Inc., San Diego, CA).

### Measurement of Cellular Impedance

Impedance measurements were performed using the CellKey system (Molecular Devices). CHO<sub>RGBR</sub> cells were seeded at a density of 30,000 cells/well into CellKey microplates (MDS SCIEX) in 100 µL of growth

medium and cultured overnight. The following day, cells were washed three times with assay buffer (HBSS containing 20 mmol/L HEPES, pH 7.4) and allowed to equilibrate for 30 min. After a 90 sec read to establish a baseline, ligands (5× in assay buffer) were added online in the CellKey instrument and the changes of cellular impedance were measured over 10 min. For the desensitization assay, cells were incubated with GABA or the PAMs for 20 min. Subsequently, the cells were washed three times with assay buffer to remove the ligand and allowed to equilibrate in assay buffer for 30 min before being stimulated a second time with a fixed concentration of GABA at an EC<sub>90</sub> (5× in assay buffer). The changes of cellular impedance induced by a second addition were measured over 10 min. The effects of GABA and the PAMs were calculated relative to the stimulation obtained with a maximally active concentration of GABA. CRCs were determined by nonlinear regression analysis using Prism software (GraphPad Software Inc., San Diego, CA).

## Results

### Effects of NVP998 on nicotine self-administration

The plasma concentrations of GS39783, BHF177, and NVP998, in rats treated with 20 mg/kg of each of the three compounds were 482 ± 83 ng/mL (1.4 ± 0.2 µmol/L), 125 ± 3 ng/mL (0.4 ± 0.008 µmol/L), and 407 ± 210 ng/mL (1.1 ± 0.6 µmol/L) at the beginning of the experiment (concentration at 1 h) and 472 ± 16 ng/mL (1.4 ± 0.04 µmol/L), 145 ± 13 ng/mL (0.4 ± 0.03 µmol/L), and 284 ± 176 ng/mL (0.8 ± 0.5 µmol/L) after testing (concentration at 2 h), respectively (Table 1). The corresponding brain concentrations of GS39783, BHF177, and NVP998, were 635 ± 114 ng/mL (1.9 µmol/L), 979 ± 80 ng/mL (2.8 µmol/L), and 321 ± 147 ng/mL (0.9 µmol/L), respectively, (Table 1). These data indicate that the GABA<sub>B</sub>R allosteric ligands displayed low micromolar plasma and brain concentration during testing. However, in contrast to BHF177

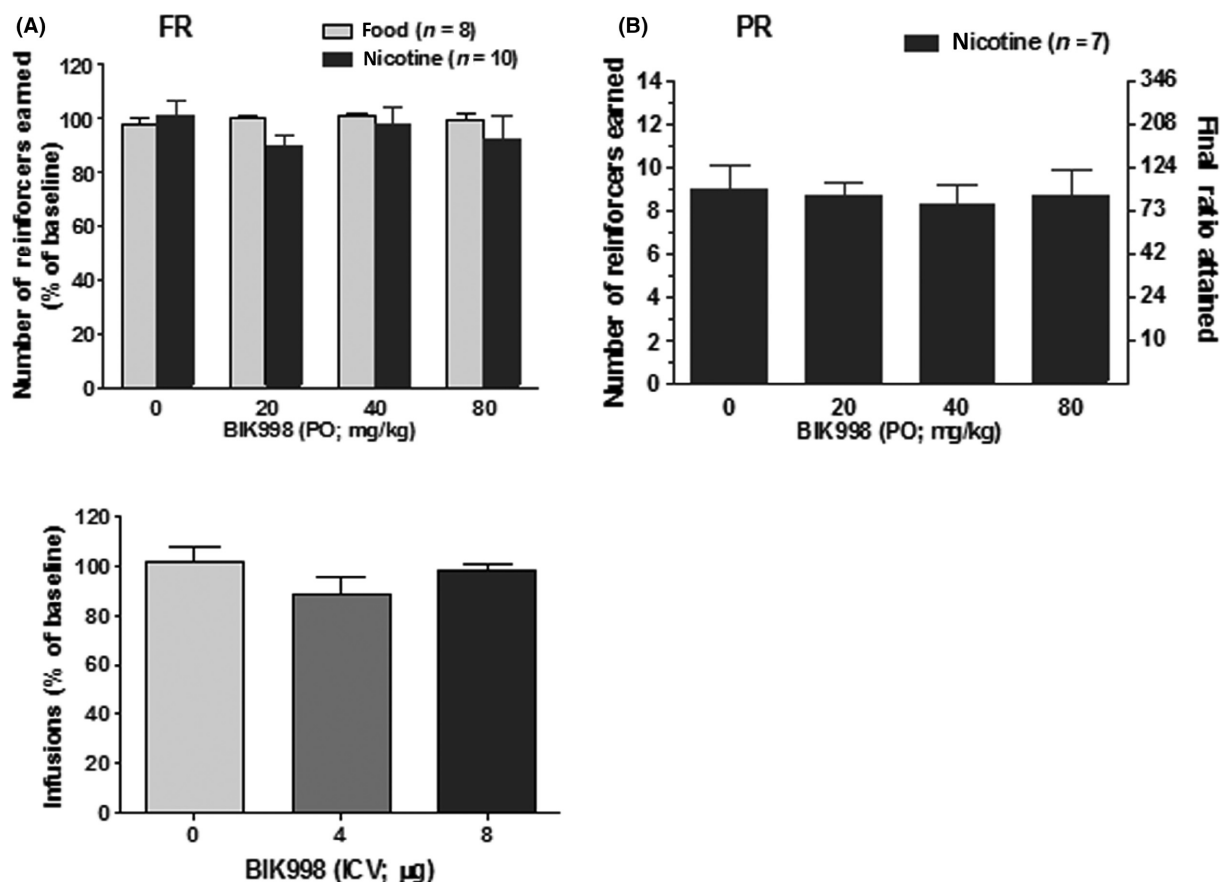
**Table 1.** Plasma and brain concentrations of NVP998, BHF177, and GS39783, before and at the end of the experiment in rat.

Drug	Route	Dose (mg/kg <sup>-1</sup> )	Plasma exposure						Brain exposure		
			Conc. 0.5 h		Conc. 1 h		Conc. 2 h		Conc. 2 h		
			ng/mL	µmol/L	ng/mL	µmol/L	ng/mL	µmol/L	ng/mL	µmol/L	µmol/L
GS39783	p.o.	20	500 ± 67	1.5 ± 0.2	482 ± 83	1.4 ± 0.2	472 ± 16	1.4 ± 0.04	635 ± 114	1.9 ± 0.3	
BHF177	p.o.	20	76 ± 44	0.2 ± 0.1	125 ± 3	0.4 ± 0.008	145 ± 13	0.4 ± 0.03	979 ± 80	2.8 ± 0.2	
NVP998	p.o.	20	425 ± 184	1.2 ± 0.5	407 ± 210	1.1 ± 0.6	284 ± 176	0.8 ± 0.5	321 ± 147	0.9 ± 0.4	

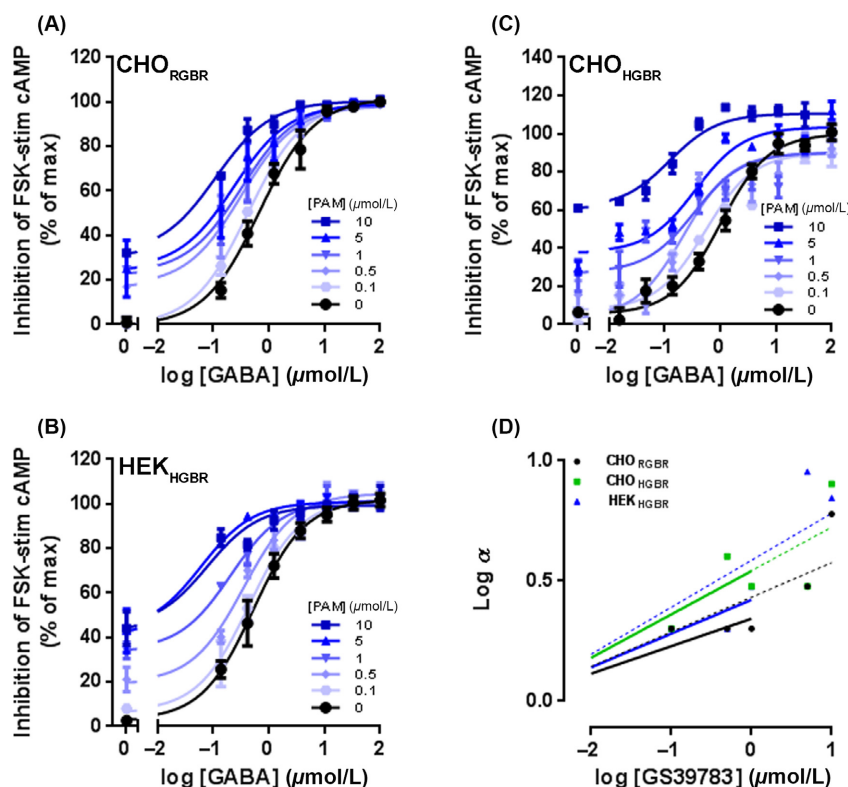
(20 mg/kg p.o.) that significantly decreased nicotine self-administration in rats (Paterson *et al.* 2008), systemic administration of NVP998 (20, 40 and 80 mg/kg, p.o.) did not affect nicotine self-administration under a fixed-ratio or a progressive-ratio schedule of reinforcement (Fig. 1A and B). The lack of effects of systemic NVP998 on nicotine self-administration might be due to poor brain exposure because this compound had the lowest brain concentration among these three PAMs. However, this possibility was excluded because i.c.v. administration of NVP998 also had no effect on nicotine self-administration (Fig. 1C). Thus, we hypothesized that the differences in drug responsiveness may result from differences in the intrinsic properties of the compound that are not observed using traditional GTP $\gamma$ S-binding assays. Therefore, we further investigated the effect of the allosteric ligands on GABA<sub>B</sub>-R-mediated, changes in intracellular cAMP levels, intracellular [Ca<sup>2+</sup>], and ERK<sub>1/2</sub> phosphorylation.

### Effects of GS39783 and analogs on GABA<sub>B</sub>-R-mediated inhibition of cAMP production

Upon GABA binding, GABA<sub>B</sub>R preferentially couples to the G $\alpha_i$  pathway, inhibiting adenylyl cyclase activity, and thus decreasing the level of intracellular cAMP. Therefore, we first determined the effects of GS39783, BHF177, and NVP998 on GABA-mediated inhibition of forskolin (FSK)-stimulated cAMP production using a CHO cell line expressing a functional GABA<sub>B</sub>R comprised of human GABA<sub>B</sub>R1b and rat GABA<sub>B</sub>R2 (CHO<sub>RGBR</sub>), or stable HEK293 and CHO cell lines expressing a functional GABA<sub>B</sub>R comprised of human GABA<sub>B</sub>R1b and human GABA<sub>B</sub>R2 (HEK<sub>HGBR</sub> and CHO<sub>HGBR</sub>, respectively). As expected, the addition of GABA resulted in a concentration-dependent inhibition of FSK-stimulated cAMP production with an EC<sub>50</sub> value of 0.67  $\mu$ mol/L, 0.52  $\mu$ mol/L, and 0.99  $\mu$ mol/L in CHO<sub>RGBR</sub>, HEK<sub>HGBR</sub>, and CHO<sub>HGBR</sub> cells, respectively (Figs. 2–4). The inhibition of cAMP



**Figure 1.** Effects of the GABA<sub>B</sub>-R-positive allosteric modulator NVP998, on nicotine self-administration in rats. Systemic administration of NVP998 (20, 40 and 80 mg/kg) had no effect on nicotine or food self-administration under fixed- or progressive-ratio schedules of reinforcement in rats. Similar lack of effect was also observed after i.c.v. administration of NVP998 (4 and 8  $\mu$ g/side).



**Figure 2.** cAMP HTRF-measured effects of GS39783 on GABA-induced GABA<sub>B</sub>-mediated inhibition of forskolin-stimulated cAMP production. Concentration–response curves for GABA in the absence and in the presence of increasing concentration of allosteric ligand in CHO<sub>RGBR</sub> (A), HEK<sub>HGBR</sub> (B), or CHO<sub>HGBR</sub> (C) cells line. (D) Linear regression plots were constructed from the logarithm of GS39783 concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ). The solid and dashed trendlines were created by plotting the  $\alpha$  values obtained with GS39783 at 0.1–1 μmol/L or 0.1–10 μmol/L, respectively. The data are means  $\pm$  SEM of a typical experiment that was performed three times.

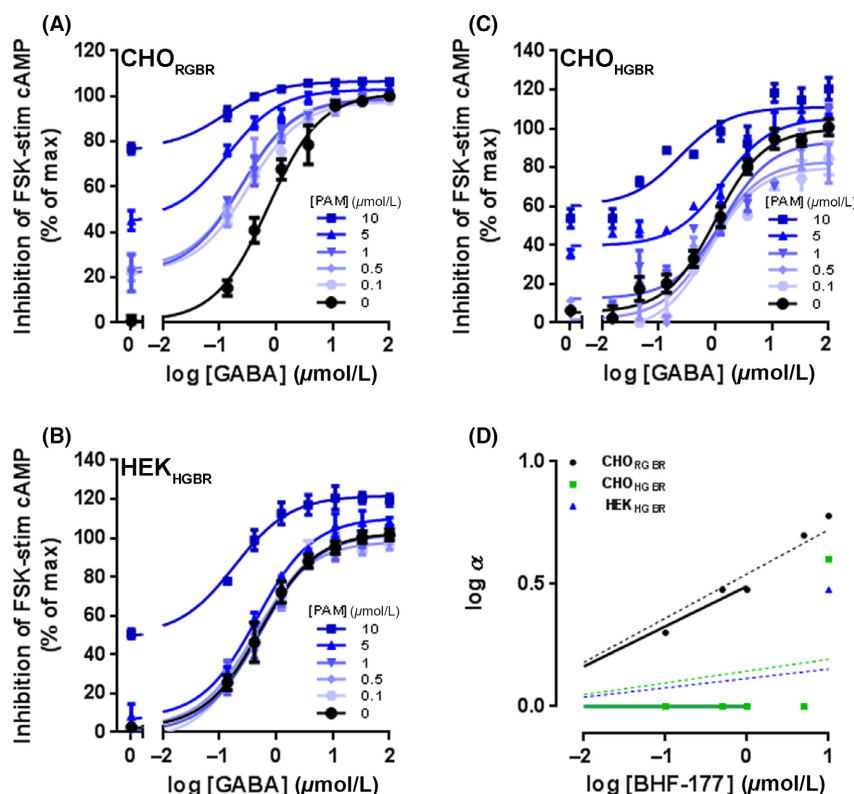
formation by GABA was fully antagonized by the competitive antagonist CGP54626, and no effect of GABA was observed in nontransfected CHO or HEK293 cells (data not shown).

Co-addition of 0.1–1 μmol/L of GS39783, resulted in a twofold leftward potency shift in GABA EC<sub>50</sub> in the CHO<sub>RGBR</sub> cells, and 5–10 μmol/L enhanced GABA EC<sub>50</sub> ~3–6 fold (Fig. 2A, Table 2). We have assigned the potency shift observed in the presence of an allosteric modulator an ' $\alpha$ ' value, where an  $\alpha$  value >1 defines positive modulation, and an ' $\alpha$ ' value <-1 defines negative modulation. Hence, in CHO<sub>RGBR</sub> cells, 10 μmol/L GS39783 has an  $\alpha$  = 6 (Table 2). In HEK<sub>HGBR</sub> and CHO<sub>HGBR</sub> cells, 0.1–1 μmol/L and 5–10 μmol/L of GS39783 generated  $\alpha$  values of ~2–3 and ~7–9, respectively (Fig. 2B and C; Table 2). Furthermore, GS39783 also exhibited significant intrinsic activity (~30% of GABA EC<sub>max</sub>) in CHO<sub>RGBR</sub>, HEK<sub>HGBR</sub>, and CHO<sub>HGBR</sub> cells when present at 0.5–10 μmol/L (Fig. 2A and B; Table 2). Correlation plots constructed from the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ) in the presence of a given concentration of the modulator demonstrated that with regard

to cAMP production, GS39783 exhibits similar PAM activity in cellular systems expressing either the rat or the human GABA<sub>B</sub>-R heterodimer (Fig. 2D, Table 3). Importantly, low micromolar concentration (0–1 μmol/L) showed significant PAM activity with  $P$  = 0.0018, 0.0023, and 0.0015 ( $F$ -test from nonzero slope) in CHO<sub>RGBR</sub>, CHO<sub>HGBR</sub>, and HEK<sub>HGBR</sub>, respectively (Fig. 2D, Table 3). These results are in good agreement with GS39783 pharmacokinetics (Table 1) and in vivo efficacy (Paterson et al. 2008).

We next evaluated BHF177 in the CHO<sub>RGBR</sub> cells. Sub-micromolar concentration (0.1–1 μmol/L) of BHF177 enhanced GABA potency by 2–3 fold ( $\alpha$  = 2–3), whereas 5 μmol/L and 10 μmol/L generated  $\alpha$  values of 5–6 (Fig. 3A, Table 2). In addition, BHF177 displayed significant intrinsic activity even at low micromolar concentrations and behaved almost as a full agonist when present at 10 μmol/L, activating the GABA<sub>B</sub>-R by ~80% of GABA EC<sub>max</sub> (Fig. 3A, Table 2). In contrast, in HEK<sub>HGBR</sub> and CHO<sub>HGBR</sub> cells, BHF177 neither modulated nor promoted GABA<sub>B</sub>-R activity when present at 0.1–1 μmol/L (Fig. 3B and C, Table 2). Nevertheless, PAM activity





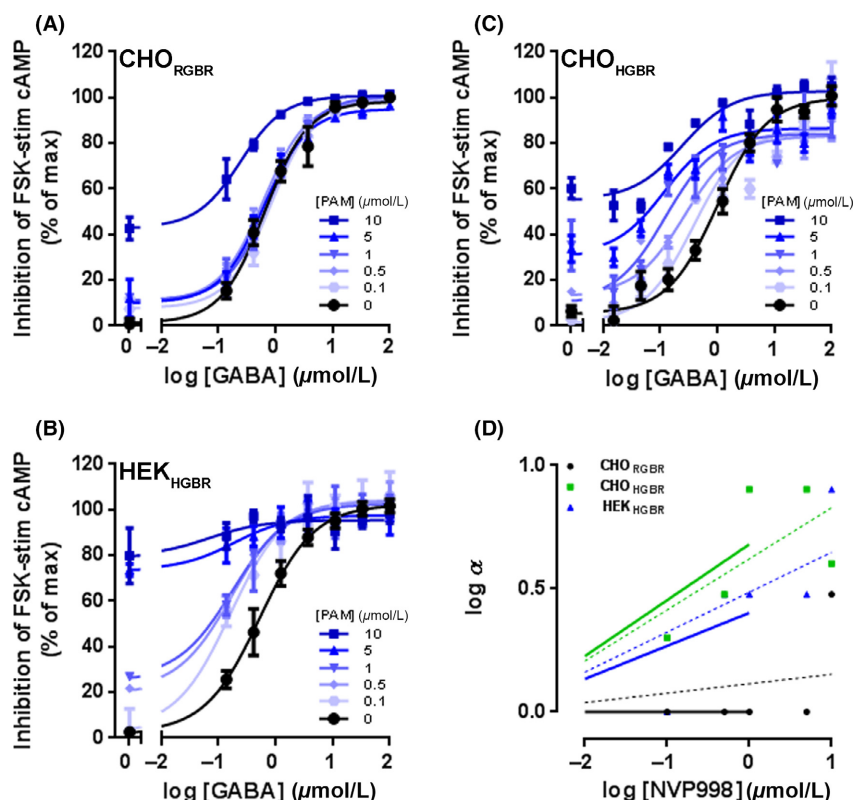
**Figure 3.** cAMP HTRF-measured effects of BHF177 on GABA-induced GABA<sub>B</sub>-R-mediated inhibition of forskolin-stimulated cAMP production. Concentration–response curves for GABA in the absence and in the presence of increasing concentration of allosteric ligand in CHO<sub>RGBR</sub> (A), HEK<sub>HGBR</sub> (B), or CHO<sub>HGBR</sub> (C) cells line. (D) Linear regression plots were constructed from the logarithm of BHF177 concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ). The solid and dashed trendlines were created by plotting the  $\alpha$  values obtained with BHF177 at 0.1–1  $\mu$ mol/L or 0.1–10  $\mu$ mol/L, respectively. The data are means  $\pm$  SEM of a typical experiment that was performed three times.

( $\alpha = 3$ –4) and significant intrinsic activity was observed at 10  $\mu$ mol/L (Fig. 3B and C, Table 2). In addition, BHF177 at 10  $\mu$ mol/L was found to significantly increase GABA efficacy to  $\sim 125\%$  in HEK<sub>HGBR</sub> (Fig. 3B; Table 2). Correlation plots constructed from the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ) in the presence of a given concentration of the modulator demonstrated that low micromolar concentration exhibit significant PAM activity with  $P = 0.0001$  ( $F$ -test from nonzero slope) in cells expressing the rat receptor (CHO<sub>RGBR</sub>) (Fig. 3D, Table 3). These in vitro data are in good agreement with BHF177 pharmacokinetics (Table 1) and in vivo efficacy (Paterson *et al.* 2008). Interestingly, similar concentrations of BHF177 did not display PAM activity in cellular systems expressing the human receptor (HEK<sub>HGBR</sub> and CHO<sub>HGBR</sub>) suggesting that BHF177 might exhibit a distinct in vitro pharmacological profile depending on the composition of the GABA<sub>B</sub>-R heterodimer.

NVP998 exhibited PAM activity ( $\alpha=3$ ) and displayed intrinsic activity ( $41 \pm 4\%$ ) when present at the higher concentration (10  $\mu$ mol/L) in the CHO<sub>RGBR</sub> cells

(Fig. 4A, Table 2). In contrast, low micromolar concentrations of this allosteric ligand (0.1–5  $\mu$ mol/L) failed to enhance GABA EC<sub>50</sub> or to activate the receptor in this cellular system (Fig. 4A and D, Table 2). These in vitro observations are in good agreement with the absence of an in vivo effect of NVP998 on nicotine self-administration (Fig. 1). However, in HEK<sub>HGBR</sub> and in CHO<sub>HGBR</sub> cells, NVP998 significantly enhanced GABA potency even when present at low micromolar concentration with  $P = 0.040$  and  $0.009$  ( $F$ -test from nonzero slope), respectively (Fig. 4B–D, Table 3). Furthermore, in these cellular systems, 5 and 10  $\mu$ mol/L of NVP998 displayed significant agonist activity (Table 2). Thus, as previously observed with BHF177, NVP998 exhibits system-dependent ago-PAM activity.

Although, these data confirm that GS39783, BHF177, and NVP998 display PAM activity at the GABA<sub>B</sub>-R; they also reveal the existence of cellular system-dependent effects suggesting that GABA<sub>B</sub>-R allosteric ligands might exhibit species selectivity. Interestingly, although NVP998 and BHF177 both display species selectivity, they do so in



**Figure 4.** cAMP HTRF-measured effects of NVP998 on GABA-induced GABA<sub>B</sub>-mediated inhibition of forskolin-stimulated cAMP production. Concentration–response curves for GABA in the absence and in the presence of increasing concentration of allosteric ligand in CHO<sub>RGBR</sub> (A), HEK<sub>HGBR</sub> (B), or CHO<sub>HGBR</sub> (C) cells line. (D) Linear regression plots were constructed from the logarithm of NVP998 concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ). The solid and dashed trendlines were created by plotting the  $\alpha$  values obtained with NVP998 at 0.1–1 μmol/L or 0.1–10 μmol/L, respectively. The data are means  $\pm$  SEM of a typical experiment that was performed three times.

favor of different species; NVP998 exhibiting increased PAM activity at the human receptor, whereas BHF177 exhibited significant PAM activity at the rat receptor (Fig 3D and 4D).

### Effects of GS39783 and analogs on GABA<sub>B</sub>-mediated mobilization of intracellular Ca<sup>2+</sup>

Previous reports have demonstrated that the GABA<sub>B</sub>R can promote increases in intracellular calcium concentrations [Ca<sup>2+</sup>]<sub>i</sub> via the activation of either inositol-3 phosphate receptors (IP<sub>3</sub>Rs) store-operated channels, or by directly interacting with voltage-gated Ca<sub>v</sub>1.3 Ca<sup>2+</sup> channel (Park et al. 2010; New et al. 2006; Meier et al. 2008). Therefore, we investigated the possibility that the aforementioned allosteric ligands can modulate GABA<sub>B</sub>-mediated increases in intracellular Ca<sup>2+</sup> levels in the HEK<sub>HGBR</sub> and the CHO<sub>RGBR</sub> cells. As expected, addition of GABA induced a rise in [Ca<sup>2+</sup>]<sub>i</sub> within 20–30 sec of GABA addition, reaching a maximal response at 45–50 sec after

agonist addition, before returning to basal levels within 120 sec (data not shown).

As shown in Figures 5–7, addition of GABA resulted in a concentration-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> with an EC<sub>50</sub> value of 1.4 and 0.25 μmol/L in the CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells, respectively. These EC<sub>50</sub> values correlate well with EC<sub>50</sub> values obtained in the cAMP HTRF assay. The GABA-induced Ca<sup>2+</sup> response was abolished by co-treatment with the competitive antagonist CGP54626 in a concentration-dependent manner and no response to GABA<sub>B</sub>R agonists was observed in the parental CHO and HEK cell lines (data not shown).

In the CHO<sub>RGBR</sub> cell line, 1, 5, and 10 μmol/L of GS39783, decreased GABA potency by two-, three-, and sixfold, respectively (Fig. 5A; Table 2). Similarly, in HEK<sub>HGBR</sub> cells, GABA EC<sub>50</sub> was decreased by threefold in the presence of 0.1 and 0.5 μmol/L of GS39783 and by 4–5 fold when the allosteric ligand was present at higher concentrations (Fig. 5B; Table 2). Thus, with regard to changes in [Ca<sup>2+</sup>]<sub>i</sub>, GS39783 decreases GABA potency

and as such behaves as a negative allosteric modulator (NAM) in both cell lines. Interestingly, in the absence of GABA, addition of 5 and 10  $\mu\text{mol/L}$  of GS39783 induced a rise in  $[\text{Ca}^{2+}]_i$  in both cellular systems ( $\sim 23\%$  and  $\sim 60\%$ , respectively) (Fig. 5A and B; Table 2). However, similar activity was also observed in parental CHO and HEK293 cells suggesting that the GS39783-induced rise in  $[\text{Ca}^{2+}]_i$  is not GABA<sub>B</sub>R specific (data not shown). As observed in the cAMP assay (Fig. 2D), correlation plots constructed from the fold shift of the GABA  $\text{EC}_{50}$  ( $\alpha$ ) in the presence of a given concentration of the modulator demonstrated that GS39783 in vitro NAM activity was comparable in CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells (Fig. 5C, Table 3). Nevertheless, low micromolar concentration (0.1–1  $\mu\text{mol/L}$ ) only exhibited significant NAM activity with  $P = 0.0009$  ( $F$ -test from nonzero slope) in cells expressing the human GABA<sub>B</sub>R heterodimer (HEK<sub>HGBR</sub>) (Fig. 5C, Table 3). Thus, together with the cAMP assay data, these observations suggest that GS39783 displays signaling pathway-specific activity.

In the CHO<sub>RGBR</sub> cells, BHF177 at 10  $\mu\text{mol/L}$  was found to exhibit PAM activity ( $\alpha = 4$ ) as well as some degree of intrinsic activity ( $12 \pm 3\%$ ), whereas lower concentrations did not significantly affect GABA<sub>B</sub>R-mediated changes in  $[\text{Ca}^{2+}]_i$  (Fig. 6A, Table 2). Conversely, in the HEK<sub>HGBR</sub> cells, BHF177 at 0.1–5  $\mu\text{mol/L}$  exhibited weak negative allosteric modulator (NAM) activity as indicated by a rightward shift of the GABA CRC (Fig. 6B; Table 2). Nevertheless, correlation plots constructed from the fold shift of the GABA  $\text{EC}_{50}$  ( $\alpha$ ) in the presence of a given concentration of the modulator demonstrated that with regard to  $[\text{Ca}^{2+}]_i$  mobilization, BHF177 does not exhibit significant PAM/NAM activity in cellular systems expressing either the rat or the human GABA<sub>B</sub>R heterodimer (Fig. 2D, Table 3). These data indicate that BHF177 has differential effects on GABA<sub>B</sub>R-mediated changes in intracellular calcium levels versus cAMP levels suggesting that BHF177 not only exhibits species selectivity but also pathway-selective behavior.

In the CHO<sub>RGBR</sub> cell line, 0.1, 0.5, and 1  $\mu\text{mol/L}$  of NVP998 decreased GABA potency by three-, four-, and sixfold, respectively (Fig. 7A; Table 2). In contrast, at 5 and 10  $\mu\text{mol/L}$ , the allosteric ligand exhibited PAM activity and enhanced GABA potency by 2–3 fold. The correlation plot constructed from the fold shift of the GABA  $\text{EC}_{50}$  ( $\alpha$ ) in the presence of a given concentration of the modulator demonstrated that at low micromolar concentrations NVP998 exhibits significant NAM activity with  $P = 0.0001$  ( $F$ -test from nonzero slope) in cells expressing the rat GABA<sub>B</sub>R receptor (Fig. 7C, Table 3). These in vitro observations correlate well with the absence of an in vivo effect of NVP998 on nicotine self-administration (Fig. 1). In HEK<sub>HGBR</sub> cells, NVP998 at 10  $\mu\text{mol/L}$  exhibited PAM activity ( $\alpha = 3$ ) (Fig. 7B; Table 2) and

displayed intrinsic agonist activity ( $\sim 20\%$ ). In contrast, lower concentrations of the allosteric ligand did not affect GABA<sub>B</sub>R activity in this cellular assay system.

### Effects of GS39783 and analogs on GABA<sub>B</sub>R-mediated ERK<sub>1/2</sub> phosphorylation

It has been reported that GABA<sub>B</sub>R activation leads to the activation of the ERK<sub>1/2</sub> signaling cascade. ERK<sub>1/2</sub> activation occurs via the GABA<sub>B</sub>R2 subunit coupling to  $G_{i/o}$  proteins by releasing the  $G_{\beta\gamma}$  subunit (Tu *et al.* 2007). In addition, a recent report indicated that the GABA<sub>B</sub>R can increase ERK<sub>1/2</sub> phosphorylation by directly interacting with, and activating the Ca(v)1.3 channel (Im and Rhim 2012). Thus, we next investigated the possibility that the allosteric ligands would also modulate GABA<sub>B</sub>R-mediated ERK<sub>1/2</sub> phosphorylation. We first studied the effect of a saturating concentration of GABA (100  $\mu\text{mol/L}$ ) on ERK<sub>1/2</sub> phosphorylation in the CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells using the “*In cell*” western blot technique to determine the time point at which maximal ERK<sub>1/2</sub> phosphorylation occurs after receptor activation. Addition of GABA induced a rapid and transient increase in ERK<sub>1/2</sub> phosphorylation peaking at 5 min, with no changes in total ERK<sub>1/2</sub> expression in both cellular systems (Fig. S1). Importantly, no response to the GABA<sub>B</sub>R agonist was observed in the parental CHO or HEK293 cell line (data not shown).

To investigate the effect of the allosteric ligands on GABA<sub>B</sub>R-mediated ERK<sub>1/2</sub> activation, both cellular systems were stimulated with GABA alone or in the presence of the modulator for 5 min, and ERK<sub>1/2</sub> phosphorylation was measured using Cellul’erk HTRF assay. As anticipated, addition of GABA resulted in a concentration-dependent increase in phospho-ERK with an  $\text{EC}_{50}$  value of 0.56  $\mu\text{mol/L}$  and 0.65  $\mu\text{mol/L}$  in CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> cells, respectively (Fig. 8). We next investigated the effect of the allosteric ligands on GABA<sub>B</sub>R-mediated ERK<sub>1/2</sub> phosphorylation.

In CHO<sub>RGBR</sub> cells, 1  $\mu\text{mol/L}$  GS39783 alone did not promote ERK<sub>1/2</sub> phosphorylation, whereas it enhanced GABA potency by fivefold when present at 10  $\mu\text{mol/L}$  (Fig. 8A; Table 2). In addition, in this cellular system, the maximal stimulation obtained by saturating concentrations of GABA was significantly increased in the presence of 1 and 10  $\mu\text{mol/L}$  of the allosteric ligand ( $\sim 120\%$ ) (Fig. 8A; Table 2). In HEK<sub>HGBR</sub> cells, GS39873 enhanced GABA potency by two- and eightfold and exhibited significant intrinsic activity, activating ERK<sub>1/2</sub> by  $52\% \pm 7$  and  $63\% \pm 14$  when present at 1 and 10  $\mu\text{mol/L}$ , respectively (Fig. 8D, Table 2). In the CHO<sub>RGBR</sub> cells, 1 and 10  $\mu\text{mol/L}$  of BHF177 enhanced GABA potency by two- and threefold, respectively (Fig. 8B; Table 2). Also, the

**Table 2.** Effects of allosteric ligands on potency and efficacy of GABA-induced GABA<sub>B</sub>R-mediated cellular responses in CHO<sub>RGBR</sub> (Rat) or HEK<sub>HGBR</sub>, CHO<sub>HGBR</sub> (Hu) cells. Concentration–response curves for GABA were determined in the absence or presence of the indicated concentration of allosteric modulator. The potency shift values ( $\alpha$ ) are indicated, and the intrinsic activity and the maximal stimulation are expressed in percent of the effects obtained with a saturating concentration of GABA (100  $\mu$ mol/L) alone.

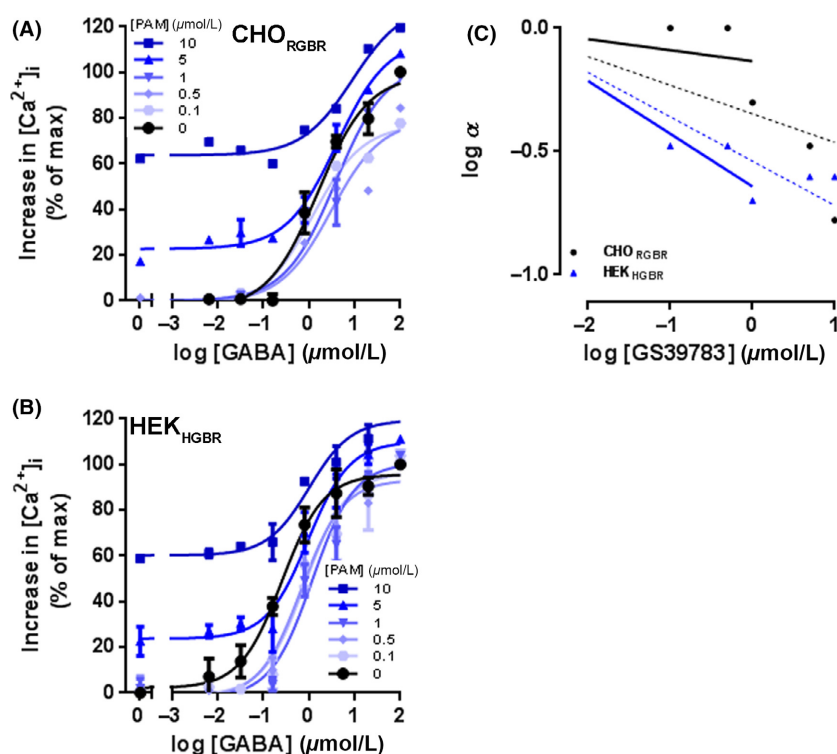
GS39783										BHF-177										NVP998												
(Drug) (μmol/L)	Int act (%)				Emax (%)				α				Int act (%)				Emax (%)				α				Int act (%)				Emax (%)			
	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu	Rat	Hu				
cAMP assay	0	1	1, 1	–1 ± 4	0 ± 5, 5 ± 2	100 ± 2	103 ± 3, 100 ± 2	1	1, 1	–1 ± 4	0 ± 5, 5 ± 2	100 ± 2	103 ± 3, 100 ± 3	1	1, 1	–1 ± 4	0 ± 5, 5 ± 2	100 ± 2	103 ± 3, 100 ± 3	1	1, 1	–1 ± 4	0 ± 5, 5 ± 2	100 ± 2	103 ± 3, 100 ± 3	1	1, 1	–1 ± 4	0 ± 5, 5 ± 2			
	0.1	2	2, 2	9 ± 3	5 ± 3, 7 ± 3	95 ± 2	101 ± 4, 90 ± 4	2	1, 1	16 ± 4*	–9 ± 4, –4 ± 5	101 ± 4	100 ± 2, 80 ± 3**	1	3, 2	8 ± 3	–3 ± 3, 1 ± 4	101 ± 1	103 ± 4, 86 ± 5	1	3, 2	8 ± 3	–3 ± 3, 1 ± 4	101 ± 1	103 ± 4, 86 ± 5	1	3, 2	8 ± 3	–3 ± 3, 1 ± 4			
	0.5	2	2, 4	28 ± 5*	17 ± 3*, 4 ± 3	95 ± 3	99 ± 4, 90 ± 4	3	1, 1	20 ± 4*	–3 ± 5, 2 ± 3	100 ± 2	99 ± 2, 83 ± 3*	1	3, 3	9 ± 5	15 ± 3, 13 ± 3	100 ± 3	102 ± 4, 83 ± 3*	1	3, 3	9 ± 5	15 ± 3, 13 ± 3	100 ± 3	102 ± 4, 83 ± 3*	1	3, 3	9 ± 5	15 ± 3, 13 ± 3			
	1	2	3, 3	35 ± 6**	31 ± 3**, 27 ± 3**	95 ± 3	102 ± 3, 92 ± 4	3	1, 1	18 ± 8	–4 ± 4, 13 ± 3	101 ± 2	103 ± 2, 93 ± 3	1	3, 8	12 ± 6	22 ± 3*, 11 ± 5	99 ± 4	100 ± 2, 84 ± 5	1	3, 8	12 ± 6	22 ± 3*, 11 ± 5	99 ± 4	100 ± 2, 84 ± 5	1	3, 8	12 ± 6	22 ± 3*, 11 ± 5			
	5	3	9, 3	29 ± 5**	32 ± 6*, 38 ± 3**	95 ± 2	98 ± 5, 110 ± 3	5	1, 1	28 ± 13	2 ± 5, 40 ± 2**	107 ± 1	111 ± 2, 105 ± 2	1	3, 8	8 ± 5	74 ± 6**, 31 ± 4**	100 ± 2	101 ± 3, 87 ± 4	1	3, 8	8 ± 5	74 ± 6**, 31 ± 4**	100 ± 2	101 ± 3, 87 ± 4	1	3, 8	8 ± 5	74 ± 6**, 31 ± 4**			
	10	6	7, 8	30 ± 4**	38 ± 4**, 61 ± 3**	96 ± 1	101 ± 2, ± 3	6	3, 4	78 ± 2**	34 ± 4**, 60 ± 5**	106 ± 3	123 ± 3**, 111 ± 4	3	8, 4	41 ± 4**	82 ± 4**, 55 ± 2**	101 ± 2	102 ± 3, 102 ± 3	3	8, 4	41 ± 4**	82 ± 4**, 55 ± 2**	101 ± 2	102 ± 3, 102 ± 3	3	8, 4	41 ± 4**	82 ± 4**, 55 ± 2**			
Calcium assay	0	1	1	–3 ± 3	1 ± 3	100 ± 4	100 ± 3	1	1	–3 ± 3	1 ± 3	100 ± 4	100 ± 3	1	1	–3 ± 3	1 ± 3	100 ± 4	100 ± 3	1	1	–3 ± 3	1 ± 3	100 ± 4	100 ± 3	1	1	–3 ± 3	1 ± 3			
	0.1	1	–3	2 ± 3	0 ± 3	74 ± 3**	96 ± 4	1	–2	–1 ± 2	–2 ± 3	89 ± 4	102 ± 5	–3	1	0 ± 2	2 ± 5	90 ± 5	100 ± 5	–3	1	0 ± 2	2 ± 5	90 ± 5	100 ± 5	–3	1	0 ± 2	2 ± 5			
	0.5	1	–3	0 ± 3	–2 ± 4	74 ± 4*	94 ± 4	2	–3	–1 ± 3	–1 ± 5	102 ± 3	105 ± 9	–4	1	–3 ± 2	–1 ± 5	94 ± 5	93 ± 5	–4	1	–3 ± 2	–1 ± 5	94 ± 5	93 ± 5	–4	1	–3 ± 2	–1 ± 5			
	1	–2	–5	2 ± 3	–2 ± 6	106 ± 5	100 ± 4	1	–2	2 ± 2	5 ± 3	98 ± 4	94 ± 4	–6	1	0 ± 2	2 ± 4	84 ± 6	100 ± 6	–6	1	0 ± 2	2 ± 4	84 ± 6	100 ± 6	–6	1	0 ± 2	2 ± 4			
	5	–3	–4	23 ± 3*	23 ± 4*	106 ± 3	110 ± 4	2	–2	–3 ± 3	–0 ± 7	101 ± 3	111 ± 10	3	1	–2 ± 3	25 ± 5*	67 ± 3**	108 ± 5	3	1	–2 ± 3	25 ± 5*	67 ± 3**	108 ± 5	3	1	–2 ± 3	25 ± 5*			
	10	–6	–4	64 ± 3**	60 ± 5**	126 ± 5*	120 ± 4*	4	1	12 ± 3*	3 ± 4	101 ± 3	100 ± 4	2	3	1 ± 3	18 ± 5*	91 ± 4	103 ± 4	2	3	1 ± 3	18 ± 5*	91 ± 4	103 ± 4	2	3	1 ± 3	18 ± 5*			
ERK assay	0	1	1	4 ± 4	2 ± 3	100 ± 4	98 ± 4	1	1	4 ± 4	2 ± 3	100 ± 4	98 ± 4	1	1	4 ± 4	2 ± 3	100 ± 4	98 ± 4	1	1	4 ± 4	2 ± 3	100 ± 4	98 ± 4	1	1	4 ± 4	2 ± 3			
	0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd			
	0.5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd			
CellKey assay	1	1	2	–3 ± 3	52 ± 7**	123 ± 5*	97 ± 7	2	5	–3 ± 4	12 ± 6	105 ± 2	102 ± 6	1	5	–6 ± 4	44 ± 6**	127 ± 2**	105 ± 6	1	5	–6 ± 4	44 ± 6**	127 ± 2**	105 ± 6	1	5	–6 ± 4	44 ± 6**			
	5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd			
	10	5	8	6 ± 2	63 ± 14**	122 ± 4*	98 ± 4	3	5	4 ± 1	30 ± 5*	117 ± 1*	106 ± 9	3	5	20 ± 1*	51 ± 5**	128 ± 1**	98 ± 9	3	5	20 ± 1*	51 ± 5**	128 ± 1**	98 ± 9	3	5	20 ± 1*	51 ± 5**			
	0	1	nd	–2 ± 1	nd	101 ± 1	nd	1	–2 ± 1	nd	nd	101 ± 1	nd	1	nd	–2 ± 1	nd	101 ± 1	nd	1	nd	–2 ± 1	nd	101 ± 1	nd	1	nd	–2 ± 1	nd			
	0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd			
	0.5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd			
CellKey assay	1	1	nd	–2 ± 2	nd	90 ± 3	nd	2	–5 ± 3	nd	nd	112 ± 3*	nd	1	nd	–2 ± 2	nd	94 ± 2	nd	1	nd	–2 ± 2	nd	94 ± 2	nd	1	nd	–2 ± 2	nd			
	5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd			
	10	15	nd	15 ± 4*	nd	118 ± 2**	nd	52	36 ± 5**	nd	nd	128 ± 5**	nd	15	nd	20 ± 3**	nd	104 ± 4	nd	15	nd	20 ± 3**	nd	104 ± 4	nd	15	nd	20 ± 3**	nd			

The data shown are means  $\pm$  SEM. Statistic by two-tailed *t*-test: \**P* < 0.05, \*\**P* < 0.01.

**Table 3.** Linear regression analyses comparing the logarithm of the allosteric ligand concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ) to predict PAM activity of GS39783 and analogs in CHO<sub>RGBR</sub>, CHO<sub>HGBR</sub>, or HEK<sub>HGBR</sub> cells. Linear regression analyses were also performed with in vivo relevant dose of the different PAMs (0–1  $\mu$ mol/L).

Assay	Cell line	(drug) ( $\mu$ mol/L)	GS39783		BHF177		NVP998	
			Slope	<i>P</i>	Slope	<i>P</i>	Slope	<i>P</i>
cAMP assay	CHO <sub>RGBR</sub>	0–1	0.1143 $\pm$ 0.0107	0.0018*	0.1637 $\pm$ 0.0059	0.0001*	–	–
		0–10	0.1440 $\pm$ 0.0166	0.0003*	0.1805 $\pm$ 0.0069	<0.0001*	0.0382 $\pm$ 0.0248	0.1855
	CHO <sub>HGBR</sub>	0–1	0.1804 $\pm$ 0.0185	0.0023*	–	–	0.2267 $\pm$ 0.0386	0.0099*
		0–10	0.1808 $\pm$ 0.0189	0.0002*	0.0482 $\pm$ 0.0314	0.1855	0.2071 $\pm$ 0.0260	0.0005*
	HEK <sub>HGBR</sub>	0–1	0.1403 $\pm$ 0.0125	0.0015*	–	–	0.1340 $\pm$ 0.0386	0.0403*
		0–10	0.1952 $\pm$ 0.0227	0.0004*	0.0382 $\pm$ 0.0248	0.1855	0.1620 $\pm$ 0.0273	0.0019*
Calcium assay	CHO <sub>RGBR</sub>	0–1	–0.0445 $\pm$ 0.0287	0.2196	0.0426 $\pm$ 0.0728	0.6176	–0.2422 $\pm$ 0.0093	0.0001*
		0–10	–0.1157 $\pm$ 0.0319	0.0152*	0.1190 $\pm$ 0.0606	0.1214	–0.0389 $\pm$ 0.0758	0.6298
	HEK <sub>HGBR</sub>	0–1	–0.2139 $\pm$ 0.0160	0.0009*	–0.1316 $\pm$ 0.0477	0.1104	–	–
		0–10	–0.1796 $\pm$ 0.0151	<0.0001*	–0.0349 $\pm$ 0.0640	0.6146	0.0382 $\pm$ 0.0248	0.1855
ERK assay	CHO <sub>RGBR</sub>	0–1	–	–	0.1003	–	–	–
		0–10	0.1118 $\pm$ 0.05931	0.2	0.1125 $\pm$ 0.0064	0.0033*	0.0763 $\pm$ 0.0404	0.2
	HEK <sub>HGBR</sub>	0–1	0.1003 $\pm$ 0.0	–	0.233	–	0.233	–
		0–10	0.1806 $\pm$ 0.04257	0.0513	0.1957 $\pm$ 0.0197	0.0101*	0.1957 $\pm$ 0.0197	0.0101*

\*Significant deviation from zero (*F*-test for non-zero slope, *P*-value).



**Figure 5.** Potentiation of GABA-induced GABA<sub>B</sub>-R-mediated intracellular calcium mobilization by GS39783. Concentration–response curves for GABA in the absence or presence of increasing concentrations of allosteric ligand in CHO<sub>RGBR</sub> (A) or HEK<sub>HGBR</sub> (B) cell lines. (C) Linear regression plots were constructed from the logarithm of GS39783 concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ). The solid and dashed trendlines were created by plotting the  $\alpha$  values obtained with GS39783 at 0.1–1  $\mu$ mol/L or 0.1–10  $\mu$ mol/L, respectively. The data are means  $\pm$  SEM of a typical experiment that was performed three times.



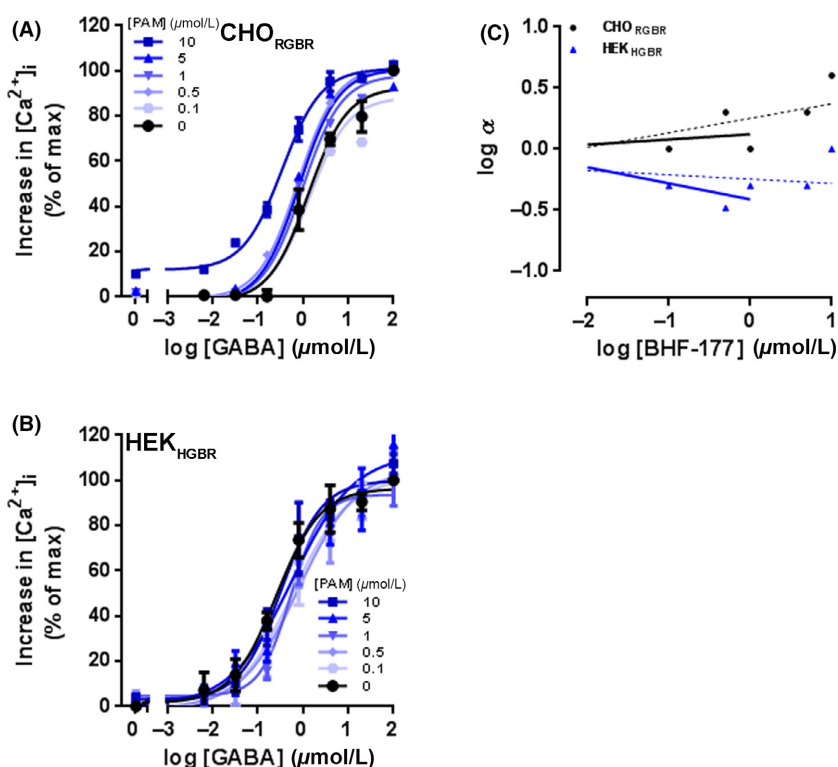
maximal stimulation obtained by a saturating concentration of GABA was significantly increased in the presence of 10  $\mu\text{mol/L}$  of BHF177 ( $117\% \pm 7$ ). In HEK<sub>HGBR</sub> cells, BHF177 enhanced GABA EC<sub>50</sub> by fivefold at 1 and 10  $\mu\text{mol/L}$ , and also displayed significant intrinsic activity at the highest concentration ( $30\% \pm 6$ ) (Fig. 8E; Table 2).

In the CHO<sub>RGBR</sub> cells, NVP998 did not enhance GABA potency at 1  $\mu\text{mol/L}$ , whereas the allosteric ligand enhanced GABA EC<sub>50</sub> by threefold at 10  $\mu\text{mol/L}$  (Fig. 5C, Table 2). In this cellular system, NVP998 also displayed significant intrinsic activity at 10  $\mu\text{mol/L}$  and activated ERK<sub>1/2</sub> by  $20\% \pm 1$  (Fig. 5C, Table 2). In addition, as observed with the other allosteric ligands, the maximal stimulation obtained by saturating concentrations of GABA was significantly increased in the presence of 1 and 10  $\mu\text{mol/L}$  of NVP998 ( $\sim 130\%$ ) (Fig. 5C, Table 2). In HEK<sub>HGBR</sub> cells, 1 and 10  $\mu\text{mol/L}$  of NVP998 enhanced GABA EC<sub>50</sub> by fivefold, and exhibited significant intrinsic activity ( $44\% \pm 6$  and  $51\% \pm 5$ , respectively) (Fig. 8F; Table 2). Correlation plots constructed from the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ) in the presence of a given

concentration of the modulator demonstrated that with regard to ERK activation, (i) BHF177 displays significant PAM activity in both CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub> with  $P = 0.0033$  and  $0.0101$  ( $F$ -test from nonzero slope), respectively, (ii) NVP998 exhibits significant PAM activity only in HEK<sub>HGBR</sub> with  $P = 0.010$  ( $F$ -test from nonzero slope), and (iii) GS39783 did not demonstrate significant PAM activity in these cellular systems (Fig. 8G, Table 3). Together with our previous observations these data confirm that GABA<sub>B</sub>R allosteric ligands not only exhibit functional selectivity but their activities are also context-dependent.

### Effects of GS39783 and analogs on GABA<sub>B</sub>R desensitization

Since the allosteric ligands display intrinsic activity, they may induce GABA<sub>B</sub>R desensitization. Thus, we further investigated the effect of GS39783, BHF177 and NVP998 on GABA<sub>B</sub>R desensitization using a label free technology (CellKey system) which allows noninvasive bioimpedance-based measurement of an integrated



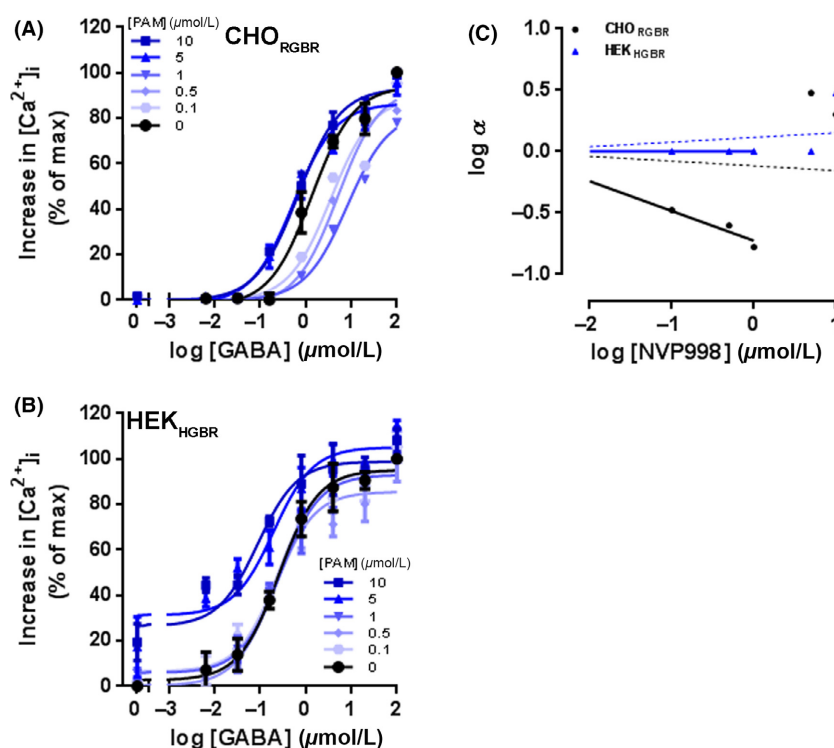
**Figure 6.** Potentiation of GABA-induced GABA<sub>B</sub>-mediated intracellular calcium mobilization by BHF177. Concentration–response curves for GABA in the absence or presence of increasing concentrations of allosteric ligand in CHO<sub>RGBR</sub> (A) or HEK<sub>HGBR</sub> (B) cell lines. (C) Linear regression plots were constructed from the logarithm of BHF177 concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ). The solid and dashed trendlines were created by plotting the  $\alpha$  values obtained with BHF177 at 0.1–1  $\mu\text{mol/L}$  or 0.1–10  $\mu\text{mol/L}$ , respectively. The data are means  $\pm$  SEM of a typical experiment that was performed three times.

cellular response (Ciambrone *et al.* 2004). In this assay, GABA induced concentration-dependent changes in cellular impedance with an EC<sub>50</sub> value of 0.57  $\mu\text{mol/L}$  in CHO<sub>RGBR</sub>. In agreement with our previous data, the allosteric ligands exhibited PAM activity and displayed intrinsic activity when present at 10  $\mu\text{mol/L}$  (Fig. 9A–C, Table 2). It is well established that following a first stimulation, GPCRs that undergo desensitization display reduced responsiveness to a second stimulation with an agonist. Therefore, CHO<sub>RGBR</sub> cells were stimulated for 20 min with 1 or 10  $\mu\text{mol/L}$  of the allosteric ligands, or 1 or 100  $\mu\text{mol/L}$  of the orthosteric agonist GABA. Subsequently, the ligand-containing medium was removed; the cells were washed three times and further incubated in assay buffer for 30 min before being stimulated a second time with a GABA EC<sub>90</sub> ( $\sim 5 \mu\text{mol/L}$ ). In CHO<sub>RGBR</sub> cells exposed to 100  $\mu\text{mol/L}$  of GABA, the response to a second stimulation was significantly attenuated (Fig. 9D). In good agreement with previous reports (Gjoni and Urwyler 2008), these data suggest that high concentrations of GABA induce GABA<sub>B</sub>R

desensitization. In contrast, in CHO<sub>RGBR</sub> cells exposed to the allosteric ligands, the variation of cellular impedance induced by the second stimulation was not decreased when compared to the control (Fig. 9E) suggesting that the allosteric ligands do not promote GABA<sub>B</sub>R desensitization.

## Discussion

In this study, we have performed extensive *in vitro* pharmacological characterization of the structurally related GABA<sub>B</sub>R-positive allosteric modulators (PAMs), GS39783, BHF177, and NVP998. We have also examined the effects of NVP998 on nicotine self-administration in the rat in order to compare these effects to our previously published data describing the effects of GS39783, and BHF177 on nicotine self-administration (Paterson *et al.* 2008; Vlachou *et al.*, 2011). The main findings are that each of the GABA<sub>B</sub>R allosteric ligands evaluated exhibit functional as well as receptor ortholog selectivity, indicating that even minor structural changes can have profound effects on

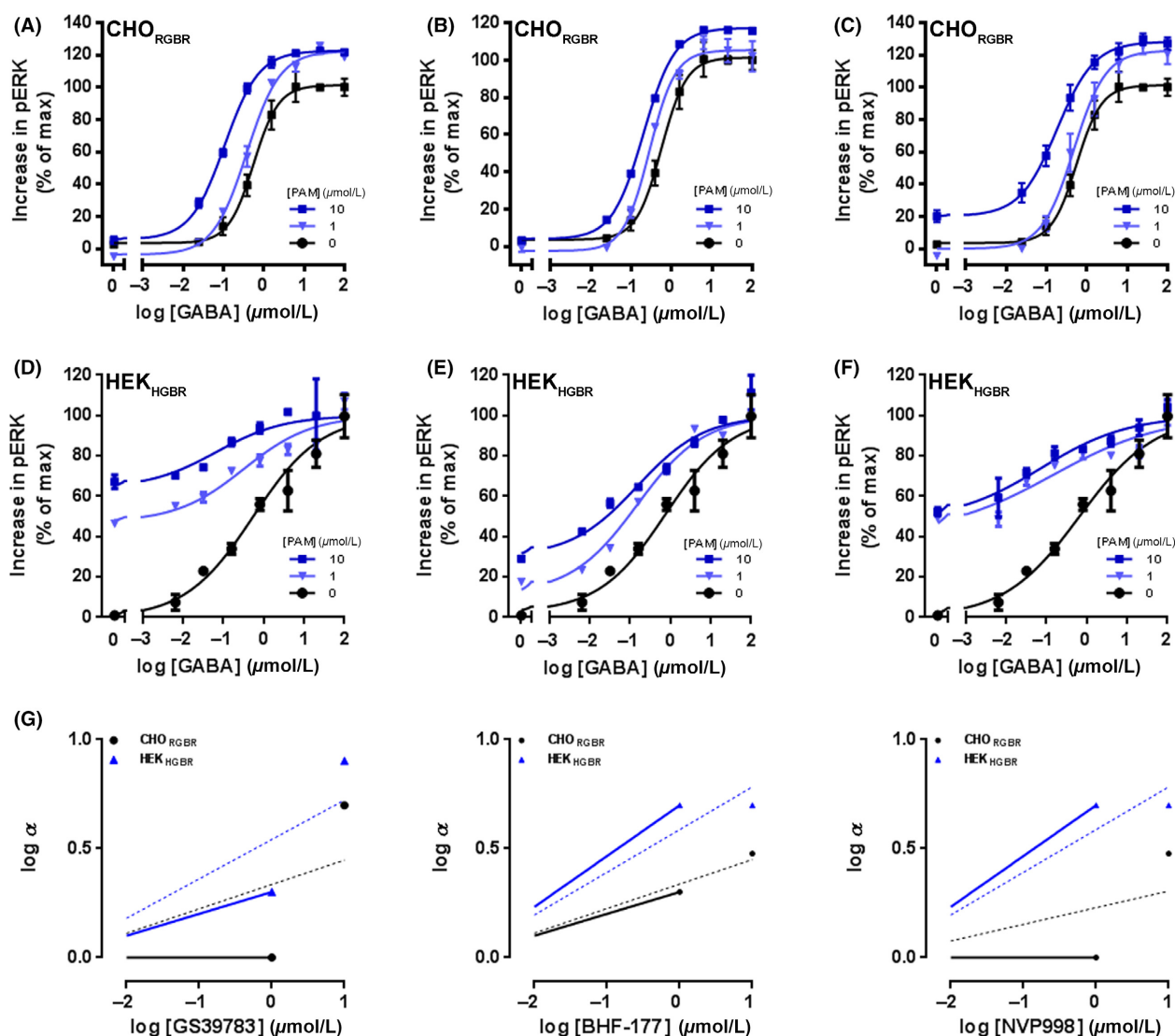


**Figure 7.** Potentiation of GABA-induced GABA<sub>B</sub>R-mediated intracellular calcium mobilization by NVP998. Concentration–response curves for GABA in the absence or presence of increasing concentrations of allosteric ligand in CHO<sub>RGBR</sub> (A) or HEK<sub>HGBR</sub> (B) cell line. (C) Linear regression plots were constructed from the logarithm of NVP998 concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> ( $\alpha$ ). The solid and dashed trendlines were created by plotting the  $\alpha$  values obtained with NVP998 at 0.1–1  $\mu\text{mol/L}$  or 0.1–10  $\mu\text{mol/L}$ , respectively. The data are means  $\pm$  SEM of a typical experiment that was performed three times.

pathway and species selectivity; critical properties that need to be considered as early as possible in the drug discovery process.

A decade ago GS39783, and structural analogs BHF177, and NVP998, were the first positive allosteric modulators (PAMs) of the GABA<sub>B</sub>R to be described. These molecules were found to potentiate GABA-stimulated guanosine 5'-O-(3-[(35S)thiotriphosphate) ([<sup>35</sup>S]GTPγS) binding to membranes generated from CHO cells heterologously expressing the human/rat GABA<sub>B</sub>R (CHO<sub>RGBR</sub>),

exhibiting maximal effect at 10 μmol/L (Urwyler et al. 2003; Guery et al. 2007). More recently, we have demonstrated that BHF177 and GS39783 are effective in decreasing nicotine self-administration in rats (Paterson et al. 2008). Interestingly, the in vivo efficacy of GS39783 was equivalent to BHF177 only when co-administered with a subeffective dose of the GABA<sub>B</sub>R agonist CGP44532. Here, we demonstrate that NVP998 when administered either p.o or i.c.v had no effect on nicotine self-administration under fixed- and progressive-ratio schedules of

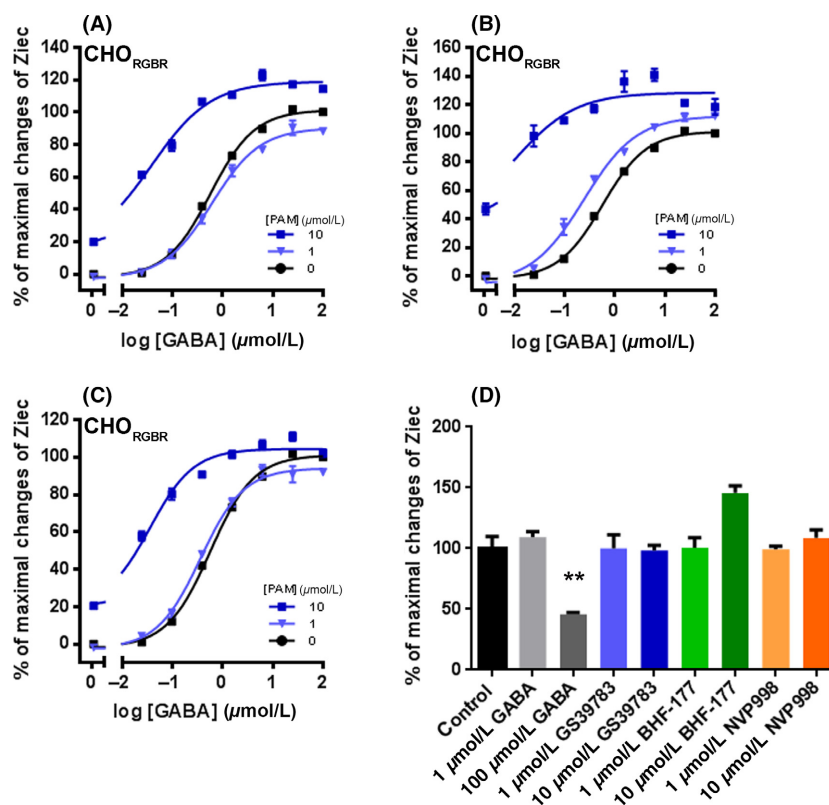


**Figure 8.** Modulation of GABA<sub>B</sub>-mediated ERK<sub>1/2</sub> phosphorylation by GS39783 and analogs. The graphs A–F show representative concentration–response curves for GABA in the Cellul'erk HTRF assay either in the absence or in the presence of 1 or 10 μmol/L of the specified allosteric ligand. The data points are normalized to the maximal effect of GABA alone. The responses were measured in either CHO<sub>RGBR</sub> (A, C, and E) or HEK<sub>HGBR</sub> (B, D, F) cell lines. (G) Linear regression plots were constructed from the logarithm of the allosteric ligand concentration against the logarithm of the fold shift of the GABA EC<sub>50</sub> (α). The solid and dashed trendlines were created by plotting the α values obtained with allosteric ligand at 0–1 μmol/L or 0–10 μmol/L, respectively. The data are means ± SEM of a typical experiment that was performed three times.

reinforcement, suggesting that this compound had no effect on the reinforcing and motivational aspects of self-administered nicotine. Thus, despite the structural similarities, reported *in vitro* PAM activity (Urwyler *et al.* 2001), equivalent oral bioavailability, and similar brain exposure (Table 1), these GABA<sub>B</sub>R PAMs exhibit distinct *in vivo* efficacies.

It is now well accepted that different ligands acting at the same receptor subtype can activate/modulate distinct patterns of downstream responses, a phenomenon referred to as “functional selectivity”, or “ligand bias”. With this in mind and as the GABA<sub>B</sub>R possesses pleiotropic signaling capacity (Bettler and Tiao 2006), we hypothesized that differences in GABA<sub>B</sub>R allosteric modulator behavior toward specific signaling pathways may account for the dissimilarities observed in their efficacy in rat model of nicotine addiction. To test this hypothesis, we characterized the *in vitro* pharmacological profile of GS39783, BHF177, and NVP998 by investigating their effect on GABA-induced GABA<sub>B</sub>R-mediated; (i) changes in intracellular cAMP levels; (ii)  $[Ca^{2+}]_i$  mobilization; and

(iii) ERK<sub>1/2</sub> phosphorylation. Since we used the rat as a preclinical model, and GS39783, BHF177, and NVP998 have previously been demonstrated to bind to the transmembrane domain of the GABA<sub>B</sub>R2 subunit (Binet *et al.* 2004), we used CHO<sub>RGBR</sub> cells expressing the human GABA<sub>B</sub>R1b and the rat GABA<sub>B</sub>R2 subunits. Importantly, the GABA half-maximal response ( $EC_{50}$ ) values obtained in the three distinct cell-based functional assays were found to correlate well with those previously reported in the literature (Urwyler *et al.* 2001). In good agreement with previous reports, 10  $\mu$ mol/L of GS39783, BHF177, and NVP998 increased GABA potency in the cAMP assay performed with CHO<sub>RGBR</sub> (Urwyler *et al.* 2003; Guery *et al.* 2007). Importantly, in this assay, BHF177 and GS39783 also displayed significant PAM activity at concentrations found in the rat brain (0.1–1  $\mu$ mol/L), enhancing GABA  $EC_{50}$  by 2–3 fold (Table 3). In contrast, NVP998, did not demonstrate PAM activity at similar concentrations. Furthermore, GS39783 and BHF177 also exhibited significant intrinsic agonist activity in this assay (Fig. 2; Table 2), whereas NVP998 did not demonstrate



**Figure 9.** Characterization of GABA<sub>B</sub>R PAM effects on receptor desensitization using the CellKey assay. Concentration–response curves for GABA in the absence or presence of GS39783 (A), BHF177 (B) and NVP998 (C) in CHO<sub>RGBR</sub>. D, Concentration–response curves for GABA in the absence or presence of increasing concentrations of allosteric ligand in CHO<sub>RGBR</sub> (A) or HEK<sub>HGBR</sub> (B) cell line. (C) Effect of GS39783, BHF177, and NVP998 on GABA<sub>B</sub>R desensitization. The data are means  $\pm$  SEM of a typical experiment that was performed three times.

ago-allosteric modulator activity. Interestingly, the CellKey assay data revealed that in contrast to the orthosteric agonist, the allosteric ligands can activate the GABA<sub>B</sub>R without inducing receptor desensitization, a phenomenon involved in the development of tolerance. The  $[Ca^{2+}]_i$  mobilization assay revealed additional differences in the behavior of the allosteric ligands in the CHO<sub>RGBR</sub> cells. Indeed, in this assay, low micromolar concentrations of GS39783 and BHF177 did not significantly affect GABA potency, whereas low micromolar concentrations of NVP998 displayed significant NAM activity resulting in a decrease in GABA EC<sub>50</sub>. Finally, in the ERK<sub>1/2</sub> activation assay, low micromolar concentrations (0.1–1  $\mu$ mol/L) of GS39783 and NVP998 did not affect GABA potency, whereas BHF177 displayed PAM activity.

These results suggest that GABA<sub>B</sub>R allosteric ligands might exhibit distinct pathway-specific activity, highlighting the complexity of allosteric ligand behavior. Furthermore, these in vitro data indicate that in contrast to GS39783 and BHF177, NVP998 at the concentrations we observed in the rat brain, do not enhance GABA-induced cAMP nor ERK signaling and negatively modulated the GABA-mediated increase in  $[Ca^{2+}]_i$ . Together with our pharmacokinetics data, the NVP998 in vitro pharmacological profile in the CHO<sub>RGBR</sub> cell line may explain the absence of effects on nicotine self-administration in rat. Thus, when comparing the in vitro profile of GS39783 and its analogs to their in vivo efficacy, our results suggest that allosteric ligands displaying an in vitro pharmacological profile similar to BHF177 may represent compounds that would be predicted to exhibit the desired in vivo properties.

In addition to functional selectivity, our studies also revealed that, GS39783, BHF177, and NVP998 may exhibit species-specific pharmacology. This characteristic has been previously described for numerous GPCR ligands including those that modulate adrenergic receptors, histamine receptors and GPR35 (Jenkins *et al.* 2012; Neetoo-Isseljee *et al.* 2013) as well as for allosteric modulators of the mGluR1 receptor, another family C GPCR (Knoflach *et al.* 2001). In agreement with previous reports demonstrating the effects of GS39783 in rat versus human GABA<sub>B</sub>R cellular systems (Urwiler *et al.* 2003), we found that this allosteric ligand displays similar efficacy in the cAMP assay in CHO<sub>RGBR</sub>, in CHO<sub>HGBR</sub>, and in HEK<sub>HGBR</sub> cells. On the other hand, BHF177 and NVP998 both displayed species-specific behaviors. Indeed, in the cAMP assay, low micromolar concentrations (0.1–1  $\mu$ mol/L) of BHF177 increased GABA potency in the CHO<sub>RGBR</sub> but not in the CHO<sub>HGBR</sub> nor in HEK<sub>HGBR</sub> cells. Interestingly, in the same assay, NVP998 displayed alternate species-selectivity. Indeed, NVP998 enhanced GABA EC<sub>50</sub> at low

micromolar concentrations and exhibited strong intrinsic activity in CHO<sub>HGBR</sub> and HEK<sub>HGBR</sub> cells, whereas its ago-PAM activity was only observed at the highest concentration tested in the CHO<sub>RGBR</sub>. Importantly, as noted above, the in vitro activity profile of these compounds together with their in vivo plasma and brain levels correlates well with their efficacy (BHF177), or lack thereof (NVP998) in a rat model of nicotine dependence. Since the in vitro pharmacological profile of NVP998 at the human receptor is comparable to that of BHF177 at the rat receptor, it may still represent a useful molecule for the development of GABA<sub>B</sub>R-targeted therapy for nicotine addiction. However, in order for a molecule like NVP998 to progress in the drug development process, it would require evaluation in an alternative preclinical model such as nonhuman primates (Le Foll *et al.*; 2007), assuming species-selectivity is not an issue.

In conclusion, our studies demonstrate that functional and species-selectivity are operational at a GABA<sub>B</sub>R allosteric site, and that these phenomena have profound implications for drug discovery targeting the GABA<sub>B</sub>R. Therefore, significant consideration must be given in the selection of appropriate ligands to progress in the drug discovery and development process. Importantly, we show that in vitro pharmacological profiling of GABA<sub>B</sub>R allosteric ligands can be predictive of in vivo efficacy in a preclinical rat model of nicotine addiction. Hence, parallel screening against receptor orthologs (human and preclinical model species) using a multi cell-based assay platform provides an ideal framework for selecting compounds with therapeutically appropriate profiles and will help bridge the gap between in vitro and in vivo compound efficacy.

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## Author Contributions

*Participated in research design:* Sturchler, Li, Markou, and McDonald. *Conducted experiments:* Sturchler, Li, Ladino. *Performed data analysis:* Sturchler, Li, Markou, and McDonald. *Wrote or contributed to the writing of the manuscript:* Sturchler, Li, Kaczanowska, Markou, Cameron, Finn, Griffin, and McDonald.

## Disclosures

None declared.



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## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Time course of endogenous ERK<sub>1/2</sub> phosphorylation after addition of 100  $\mu$ mol/L of GABA in CHO<sub>RGBR</sub> and HEK<sub>HGBR</sub>.

**Figure S2.** Sequence alignment of rat and human GB2R subunits. Mismatch between amino acids sequences are marked in red.

**Figure S3.** Chemical structures of GS39783 and its analogs, NVP-998 and BHF177.